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Immunofluorescence Investigations on Neuroendocrine Secretory Protein 55 (NESP55) in Nervous Tissues

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Cover picture

Confocal images showing the intracellular distribution of NESP55-IR (green), as compared to TGN38-IR (red), in preganglionic sympathetic neurons (top panel) and spinal motoneurons (lower panel) in the rat.

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ABSTRACT

The chromogranin family is a group of acidic, soluble, and heat-stable proteins widespread in various neuronal, neuroendocrine and endocrine tissues, where they are subcellullarly located in the secretory granules, participating in the formation of the granules. Extracellularly, chromogranins may act as protein precursors, proteolytically processed to various small bioactive peptides. Neuroendocrine secretory protein 55 (NESP55) is the most recently identified member of the chromogranin family. It is structurally related to other chromogranins. However, the biological similarity between NESP55 and its siblings has not been firmly established yet, and knowledge about NESP55 is still limited compared with other chromogranins. In the present study, we focused on the distribution and localization of NESP55 in a number of neuronal tissues using immunohistochemistry. Furthermore, the peripheral projections of NESP55 containing sympathetic postganglionic cells were investigated.

In the CNS-derived CAD cell line, NESP55, like other peptides/chromogranins, was expressed in the cell body and the long processes in a granular pattern. In addition, NESP55-IR was distinctly observed in fringe-like short processes around the cell body and along the long processes. GAP43-IR, a protein highly associated with outgrowth of neurites and development, partially overlapped with NESP55-IR in this structure. In the autonomic nervous system, NESP55 was expressed in a subpopulation of the principal neurons in all rat sympathetic ganglia studied. In the SCG, NESP55 containing neurons were found to project to the submandibular gland, the cervical lymph nodes, the iris, and the forehead skin. Some of these target-projecting neurons contained also NPY-IR, a peptide with vasoconstriction effects. The NESP55 containing SG neurons were observed to project to the forepaw pad. Among these paw pad-projecting neurons, a subpopulation contained CGRP-IR (a peptide with sudomotor effects). A subpopulation, which expressed NPY-IR, was also observed. In the rat spinal cord, NESP55-IR was found in various spinal neurons throughout the lamina IV-X, including motoneurons, autonomic sympathetic/parasympathetic neurons, interneurons and the LSN. Many of these NESP55 containing neurons were also immunoreactive to ChAT, a cholinergic marker. The lamina I-III and the sensory dorsal root ganglion lacked NESP55-IR.

The intracellular distribution of NESP55-IR in the spinal motoneurons appeared different from that in the sympathetic neurons. In the spinal motoneurons, NESP55-IR, with an appearance of dust-like particles, was observed diffusely present in the whole cytoplasm; in contrast, in the sympathetic neurons, NESP55-IR appeared to be stored in large granules, restricted to the perinuclear region of the ganglionic cells, and overlaping with the Golgi marker, TGN38.

In conclusion, the present study demonstrated that NESP55 was expressed in different functional groups of neurons in the rat sympathetic ganglia and in the spinal cord. The expression of NESP55 in the CAD cells was exceptional. Our findings may add information about this novel protein and further our understanding of its functional significance. Moreover, the finding of the striking difference in the intracellular distribution of NESP55-IR in motoneurons versus autonomic neurons supports the previous suggestion that NESP55 may be involved in both constitutive and regulated secretory pathways.

Keywords*: chromogranins, neuropeptides, secretory pathway, the CAD cell line, rat, spinal cord, sympathetic ganglia, retrograde tracing, confocal microscopy.*

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ABBREVIATION

ANS	autonomic nervous system
CAD	Cath.α (cell line)-differentiated
CgA	chromogranin A
CgB	chromogranin B
CgC/SgII	chromogranin C/Secretogranin II
CGRP	calcitonin gene-related peptide
ChAT	choline-acetyl transferase
CLSM	confocal laser scanning microscope
CNS	central nervous system
CSF	cerebrospinal fluid
ER	endoplasmic reticulum
FG	Fluoro-Gold
GAP43	growth-associated protein 43
GFAP	glial fibrillary acidic protein
IML	intermediolateral cell column
IR	immunoreactivity
LDCV	large dense cored vesicle
LSN	lateral spinal nucleus
mRNA	messenger RNA
NESP55	neuroendocrine secretory protein 55
NPY	neuropeptide Y
NeuN	neuron-specific nuclear protein
PBS	phosphate-buffered saline
PC1	prohormone convertases 1
PC2	prohormone convertases 2
PF	paraformaldehyde
PFM	protein free medium
PNMT	phenylethanolamine-N-methyltransferase
PNS	peripheral nervous system
PTH	parathyroid hormone
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
SCG	superior cervical ganglion
SCM	serum containing medium
SD	standard deviation
SDS	sodium dodecylsulfae
SG	stellate ganglion
SgIII, IV	secretogranin III (1B1075)
SgIV	secretogranin IV (HISL-19)
SgV	secretogranin V (7B2)
SgVI	secretogranin VI (NESP55)
SgVII	secretogranin VII (VGF)
SN	secretoneurin
SP	substance P
TGN38	trans-Golgi network 38
TH	tyrosine hydroxylase
VIP	vasoactive intestinal peptide

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Yongling Li**, Linda Xiu-e Hou, Annika Aktiv and Annica Dahlström (2005). Immunohistochemical characterization of differentiated CAD cells: expression of peptides and chromogranins. **Histochem Cell Biol** 124(1): 25-33.

II. **Yongling Li**, Zhanyou Wang and Annica Dahlström (2007). Neuroendocrine secretory protein 55 (NESP55) immunoreactivity in male and female rat superior cervical ganglion and other sympathetic ganglia. **Auton Neurosci** 132(1-2): 52-62.

III. **Yongling Li** and Annica Dahlström. Peripheral projections of NESP55 containing neurons in rat sympathetic ganglia. **Auton Neurosci** (Accepted).

IV. **Yongling Li**, Reiner Fischer-Colbrie and Annica Dahlström (2008). Neuroendocrine secretory protein 55 (NESP55) in the spinal cord of rat: An immunocytochemical study. **J Comp Neurol** 506(4): 733-44.

INTRODUCTION

Chromogranins

Chromogranin family members

The chromogranins constitute a family of acidic secretory proteins which share similar features both structurally and biologically. The first member, Chromogranin A (CgA), was discovered, forty years ago, in bovine chromaffin granules of the adrenal medulla (Blaschko et al., 1967; Schneider et al., 1967). The early understanding of this protein is highly associated to sympathetic neurons (De Potter et al., 1970; Bartlett et al., 1976) and to the adrenal medulla (Smith & Kirshner, 1967), assuming that it was an adrenergic protein involved in catecholamine storage (De Potter et al., 1970; O'Connor et al., 1982). Fifteen years later, when Cohn and his colleagues (Cohn et al., 1982) discovered that CgA was indeed the same protein as secretory protein I in the parathyroid gland, knowledge of this protein was greatly extended to realizing that CgA also plays an important role in the endocrine system. It was found to widely distribute in various endocrine tissues as well as in endocrine tissue tumors (O'Connor, 1983; Cohn et al., 1984; Fischer-Colbrie et al., 1985). In the meantime, evidence for the presence of CgA in cholinergic and other peptidergic neurons also emerged (Volknandt et al., 1987; Booj et al., 1989).

When CgB (Fischer-Colbrie & Frischenschlager, 1985), the second member, and secretogranin II (SgII, sometimes called Cg C) (Fischer-Colbrie et al., 1986), the third member of the chromogranin family, were characterized, it was clear from the beginning that these two proteins are structurally related to CgA, and similarly distributed in a wide range of neuronal and neuroendocrine tissues (Winkler & Fischer-Colbrie, 1992). CgA, CgB and SgII are so far the most intensively studied proteins in the chromogranin family, and, are defined as the "classic chromogranins" (Taupenot et al., 2003).

Five other acidic secretory proteins were also proposed for membership in the chromogranin family (Helle, 2004). These are SgIII (1B1075) (Ottiger et al., 1990), SgIV (HISL-19) (Krisch et al., 1986), SgV (7B2) (Marcinkiewicz et al., 1985), SgVI (NESP55)

(Ischia et al., 1997), and SgVII (the nerve growth factor inducible protein VGF) (Levi et al., 1985).

Structural properties

Chromogranins are hydrophilic and heat-stable proteins containing approximately180-700 amino acid residues with a high proportion, about 16-25%, of acidic residues (Taupenot et al., 2003). The amino acid sequences of chromogranins are well conserved among mammalian species. The chromogranins are capable of undergoing some post-translational modifications, such as glycosylation, phosphorylation, sulfation, and proteolytic processing, etc (Winkler & Fischer-Colbrie, 1992). Due to the acidic, hydrophilic nature and the post-translated proteoglycan forms of chromogranins, which cause reduced dodecylsulfate (SDS) binding and retardation in SDS gels, the molecular weight of these proteins observed in SDS-gel electrophoresis always show a value considerably higher than that calculated from the primary sequence (Simon & Aunis, 1989; Winkler & Fischer-Colbrie, 1992; Taupenot et al., 2003; Eder et al., 2004).

Dibasic cleavage sites are frequently present in the chromogranin molecules (Taupenot et al., 2003). The most cleavage sites were found in the CgB sequence, containing 16 pairs (Benedum et al., 1987) and the least in 7B2, having 4 pairs (Mbikay et al., 2001). At these sites chromogranins are proteolytically processed to small, probably bioactive, peptides by various colocalized enzymes, such as prohormone convertases 1 and 2 (PC1 and PC2) (Seidah et al., 1990; Winkler & Fischer-Colbrie, 1992; Dillen et al., 1993; Laslop et al., 1998; Fischer-Colbrie et al., 2002). A potential disulfide-bonded loop is present in some of these molecules at the N-terminal region (Helle, 2004). Some of the chromogranins are also able to bind calcium at low pH conditions. Such structural properties, essential for the formation of secretory granules, have been characterized in the sequences of CgA, CgB, SgII and 7B2, (Helle, 2004).

Tissue distribution and subcellular localization

The distribution of chromogranins and their breakdown products is a subject extensively studied by means of different techniques such as radiommunoassy (RIA), immuno-

histochemistry, immunoelectron microscopy, immunoblot, and *in situ* hybridization. Their widespread distribution within the endocrine, neuroendocrine, as well as in the central and peripheral nervous systems, is now firmly established. Chromogranins are present in the adrenal medulla, pituitary, brain, pancreas, parathyroid, hypothalamus and in a large number of neuroendocrine/endocrine tumors (Winkler & Fischer-Colbrie, 1992; Taupenot et al., 2003; Helle, 2004). In these tissues, the subcellular localization of chromogranins was studied by means of subcellular fractionation and immuno-histochemistry at the ultrastructural level. Chromogranins are located, together with neurotransmitters and peptides/hormones, in large dense core vesicles (LDCVs), hormone storage granules, or, in the case of the adrenal medulla, in chromaffin granules (Winkler & Fischer-Colbrie, 1992).

Intracellular and extracellular functions

Chromogranins, together with neurotransmitters/hormones, are located in secretory granules of various endocrine and neuronal tissues, as discussed above, where chromogranins may act intracellularly as inducers, or helpers, in the process of sorting and packaging of chromogranins and peptides/hormones from the trans-Golgi network (TGN) to secretory granules, mainly routed to the regulated pathway (Simon & Aunis, 1989; Chanat et al., 1991; Ozawa & Takata, 1995; Laslop & Mahata, 2002). The disulfide-bonded loop present at the N-terminal region of chromogranin sequences, as well as the calcium-binding property, were considered to contribute to this process (Gerdes et al., 1989; Chanat et al., 1994; Huttner & Natori, 1995; Glombik et al., 1999; Yoo & Lewis, 2000; Yoo et al., 2001; Kim et al., 2002). Interestingly, in endocrine GH4C1 cells, removing the C-terminal 90 amino acids of CgA caused sorting of this peptide to the constitutive secretory pathway. Furthermore, as compared with wild-type chromogranin A, the aggregation properties were clearly impaired. Thus, chromogranins contain independent N- and C-terminal sorting domains that function in a cell type-specific manner (Cowley et al., 2000).

An extracellular function of chromogranins has also been suggested. Small peptides derived from chromogranins were found in various tissues, like their precursors.

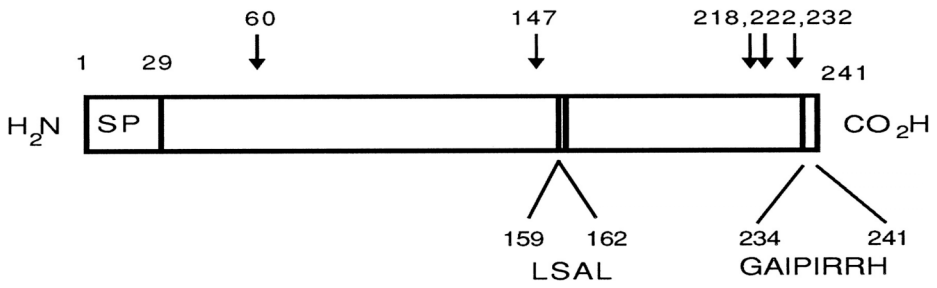

Fig. 1. Schematic presentation of bovine NESP55. The arrows indicate pairs of basic amino acids suitable for cleavage by kex-like prohormone convertases, and the numbers show the positions in the sequence. Two putative peptides generated proteolytically from NESP55, GAIPIRRH and LSAL, are indicated. SP, signal peptide (Adapted from (Ischia et al., 1997).

These peptides have been proposed to exhibit autocrine, paracrine or endocrine activities (Natori & Huttner, 1994; Taupenot et al., 2003; Helle, 2004). Some examples are bacteriolytic and antifungal effects (Metz-Boutigue *et al*., 2000), inhibition of neurotransmitter/hormone release (Russell et al., 1994), triggering of apoptotic degeneration of cortical neurons (Taupenot et al., 1996; Ciesielski-Treska et al., 2001), and other effects. The signaling pathways, by which these small peptides function, are also a hot topic for researchers. Catestatin seems to act by binding directly to the nicotinic cholinergic receptor, inhibiting catecholamine release from pheochromocytoma and adrenal chromaffin cells, as well as from noradrenergic neurites (Mahata et al., 1997; Mahata et al., 1999). Secretoneurin (SN), a 33-amino acid peptide derived from SgII, was found to interact with specific cell surface binding sites on human monocytes to induce monocyte migration (Kong et al., 1998). G-protein coupled signaling pathways were also suggested for SN and CgA-derived pancreastatin, inducing various effects in neuronal and endocrine tissues (Sanchez-Margalet et al., 2000; Fischer-Colbrie et al., 2005). However, for most chromogranin fragments, unique/specific receptors mediating these diverse responses have not yet been identified.

Neuroendocrine secretory protein 55 (NESP55)

NESP55 is the youngest member of the chromogranin family, first discovered in the bovine adrenal medulla in 1997 (Ischia et al., 1997). It shares the properties with other members described above, but it is unique due to its genomic feature, its subcellular distribution and secretion manners.

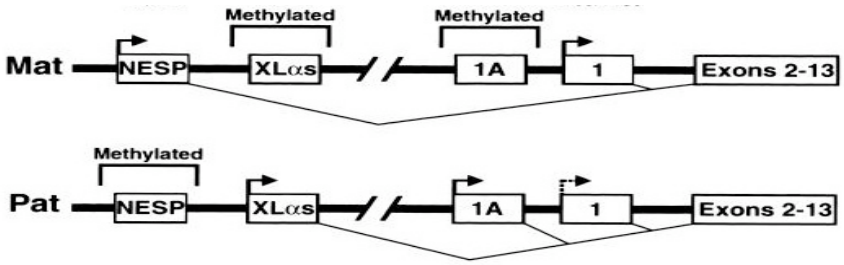

Fig. 2. Both GNAS1 and Gnas have multiple oppositely imprinted transcripts. Schematic diagram showing the maternal (Mat) and paternal (Pat) alleles of Gnas. Alternative first exons which splice into exon 2 to generate alternative mRNAs encoding NESP55, XLαs, an unknown gene product, and $G_s\alpha$ are shown as boxes labeled NESP, XLαs, 1A, and 1, respectively. Transcriptional active promoters are designated by horizontal arrows, and regions of differential methylation are outlined above each allele. Dashed horizontal arrow for exon 1 in the far right paternal allele indicates that this promoter is active in some tissues and inactive in other tissues (Reproduced and modified from Liu et al. (Liu *et al.*, 2000).

Molecular structure and genomic organization

Like other chromogranin members, NESP55 is a soluble, heat stable and acidic (21% acidic amino acids) protein. The NESP55 sequence, containing 241 amino acids, is highly conserved, with approximately 70-90% homology, among mammalian species, especially, in the N-terminal part (Weiss et al., 2000). There are 6 pairs of basic amino acids in the NESP55 sequence (Fig. 1). Post-translational modifications, such as proteolytic processing and glycosylation, also take place within the NESP55 molecule (Ischia et al., 1997; Weiss et al., 2000). Therefore, NESP55 migrates in SDS gels as a broad band with an apparent molecular weight of 55 kDa, but its molecular mass, calculated from the primary amino acid sequence, is 27-29 kDa.

NESP55 gene is part of the GNAS1 gene located on human chromosome 20q13 and mouse distal chromosome 2 (Fischer-Colbrie et al., 2002). The GNAS1 locus shows a complex pattern of genomic imprinting. It has at least four alternative promoters and first exons to generate mRNAs for NESP55, XLαs, an unknown gene product and $G_s\alpha$ (Fig. 2). The promoter of $G_s\alpha$ is unmethylated, thus $G_s\alpha$ is expressed, at least in most tissues, from both parental alleles (Liu et al., 2000). The exons encoding XLαs and the unknown product of GNAS1 show methylation on maternal alleles and are transcribed exclusively from paternal allele (Hayward et al., 1998a). In contrast, NESP55 is paternally imprinted

and expressed from the maternal allele only (Hayward et al., 1998b). The mechanism by which imprinting is generated and maintained has not been established yet.

Tissue distribution

NESP55 is widespread in endocrine, neuroendocrine and neuronal tissues. In various bovine tissues studied by RIA (Lovisetti-Scamihorn et al., 1999a), the highest amounts of NESP55 were detected in the adrenal medulla (2300 fmol/mg), followed by the pituitary, the pars anterior (223 fmol/mg) and pars posterior (42.7 fmol/mg), respectively. The kidney medulla contained 20.4 fmol/mg of NESP55. Different brain regions also expressed NESP55 with the highest concentration in the caudate nucleus (8.6 fmol/mg), followed by the hypothalamus (7.5 fmol/mg). Only small amounts were detected in the gastrointestinal tract. Body fluids such as cerebrospinal fluid (CSF), serum and urine also contain considerable amounts of NESP55. By RIA, NESP55 was not detected in the bovine spleen, pancreas, liver, testis, etc (Lovisetti-Scamihorn et al., 1999a). In contrast, positive support for the synthesis and presence of NESP55 in these tissues was obtained by RT-PCR (Khatib, 2004). In bovine adrenal medulla, NESP55-IR (immunoreactivity) appears to be preferentially localized, by immunohistochemistry, in adrenaline-synthesizing PNMT positive cells (Bauer et al., 1999b; Li et al., 2002).

Using *in situ* hybridization with specific ^{35}S-labelled oligonucleotides, localization of NESP55 messenger RNA (mRNA) in the rat brain was investigated (Bauer et al., 1999a). Considerable amounts of NESP55 mRNA were found in different brain areas including forebrain, midbrain, pons, and medulla oblongata, as well as in the spinal cord. The NESP5 mRNA was especially concentrated over noradrenergic, serotonergic and dopaminergic nuclei. Cortical areas, hippocampus, cerebellum and dorsal horn of the spinal cord appear devoid of NESP55 mRNA.

In the peripheral nervous system, NESP55-IR was observed in the rat superior cervical ganglion (SCG), the sciatic nerve (by immunofluorescence), as well as in vas deferens and splenic nerve (by RIA). NESP55 is rapidly and anterogradely transported by axonal transport (Li et al., 2002).

Proteolytic processing

Prohormone convertases PC1 and PC2 are potential endopeptidases for NESP55, and their activity may result in the liberation of intermediate and small size peptides from NESP55 at its cleavage sites. Two putative peptides, the octapeptides GAIPIRRH located at the C-terminus of NESP55 and the tetrapeptide LSAL in the center of the sequence may be the proteolytic products of bovine NESP55. GAIPIRRH was isolated previously in a random search of novel peptides secreted from bovine chromaffin granules (Sigafoos et al., 1993). LSAL, sometimes called 5-hydroxytryptamine-moduline, has been characterized as an endogenous antagonist of the serotonergic 5-HT_{1B} receptor (Bentue-Ferrer et al., 1998). However, in man, mouse and rat, both GAIPIRRH and LSAL were found to be mutated to GPIPIRRH and LHAL, respectively (Ischia et al., 1997; Weiss et al., 2000). Generation of GAIPIRRH by PC1 and PC2 has been examined in an *in vitro* study showing that both PC1 and PC2 can produce GAIPIRRH, however, PC1 being more potent than PC2 (Laslop et al., 2000).

Proteolytic processing of NESP55 to GAIPIRRH/GPIPIRRH, as studied by fractionation of tissue extracts followed by RIA, varies between tissues and species. In bovine tissues, more than 80% of NESP55 is processed to GAIPIRRH in the posterior pituitary, jejunum and colon. A comparatively less processing, about 40%, was observed in the pituitary stalk and the adrenal medulla. In contrast, very little, if any, processing apparently takes place in the brain, serum, CSF, and urine (Ischia et al., 1997; Lovisetti-Scamihorn et al., 1999a). In the posterior pituitary of man, GPIPIRRH is the dominant molecular form of NESP55 (Fischer-Colbrie et al., 2002), like in the bovine posterior pituitary. The GPIPIRRH dominance, more than 80%, is also true for the human anterior pituitary. However, in the bovine and rat anterior pituitary less than 20% of NESP55 is metabolized to GAIPIRRH/GPIPIRRH (Lovisetti-Scamihorn et al., 1999a; Weiss et al., 2000; Fischer-Colbrie et al., 2002).

The processing of NESP55 has been shown to increase greatly in the distal/terminals of the pig splenic nerve, compared with the proximal part (Lovisetti-Scamihorn et al.,

1999b). In agreement, the octapeptide GPIPIRRH appears to be the dominant component of NESP55 metabolism in the rat vas deferens (Li et al., 2002). Crush operation of the rat sciatic nerve leads to significant accumulation of GPIPIRRH in the proximal part close to the crush sites of the nerve (Li et al., 2002), suggesting a processing of axonally transported and accumulated granular NESP55 to GPIPIRRH.

Subcellular distribution and secretion

The subcellular distribution of NESP55 in bovine adrenal medulla (Ischia et al., 1997), as well as in the splenic nerve (Leitner et al., 1999), was investigated using subcellular fractionation, followed by RIA. These studies showed, by comparing the subcellular distribution of NESP55 with that of several other established granule/LDCVs markers, such as SgII and PC2, that NESP55, like other chromogranins, was located in chromaffin granules/LDCVs. This suggests that NESP55 is secreted via the regulated pathway from these cells. However, in the mouse corticotropic AtT-20 cells, NESP55 has been shown to be stored in a population of vesicles with a slightly lighter density than, but partially overlapping with, the vesicles of the regulated pathway. In this cell line NESP55 was apparently routed primarily to the constitutive pathway and was continuously secreted to the cell culture media, while only a minor fraction of NESP55 was sorted to the regulated pathway and released upon stimulation (Eder et al., 2004). Thus, NESP55 may be involved in both regulated and constitutive secretion.

AIMS

The purpose of this study was to investigate the distribution and localization of NESP55 immunoreactivity in neuronal tissues, and furthermore to study the peripheral projections of NESP55 containing sympathetic neurons.

The specific aims were:

1. To investigate the expression of chromogranins/NESP55 in a CNS-derived cell line, the CAD cell line, after neuronal differentiation induced by protein starvation.

2. To investigate the expression of NESP55 in rat sympathetic ganglia.

3. To investigate the peripheral projections of NESP55 positive neurons in rat superior cervical ganglion and stellate ganglion.

4. To map the distribution and localization of NESP55 in rat spinal cord.

MATERIALS AND METHODS

Cell culture (Paper I)

CAD cells (generously donated by Dr. James K. T. Wang at Tufts University School of Medicine, Boston, USA) were plated at a density of $2.0x10^4$ cells/ml in 35 mm diameter tissue culture dishes containing a serum-containing medium (SCM). One day later, cells were divided into two groups: one group was switched to a protein free medium (PFM), and the second group maintained in the SCM. After 5 days in the PFM (differentiated CAD cells) or 3 days in the SCM (undifferentiated CAD cells), the cells were fixed in 4% paraformaldehyde (PF, pH 7.4) for 20 minutes, then processed for immunohistochemical study.

Potassium stimulation of CAD cells (Paper I)

In order to investigate whether the differentiated CAD cells have capacities of exo-/endocytosis, two separate experiments of K^+ stimulation were carried out. In the first experiment, cells was stimulated with KCl (60-120 mM) for 3, 5, 10, 15, or 30 minutes, rinsed and fixed in 4% PF, then processed for immunostaining with anti-NPY and the secondary antibody (FITC labeled anti-rabbit-IgG). Any changes in immunoreactivity intensity in the cells were studied. The second experiment was designed to investigate exocytosis coupled endocytosis. Cells were stimulated with KCl (60-120 mM) in a medium containing rabbit-anti-NPY (1:800) for 10, 30 seconds, 1, 3, or 5 minutes before fixation. Incubation with FITC labeled anti-rabbit-IgG was carried out to visualize any endocytotic uptake of the NPY-antibody in the medium, which was trapped into vesicles during endocytosis. Control cultures were stimulated in potassium free medium.

Animals (Papers II, III and IV)

Adult (9-11w) Sprague-Dawley rats purchased from B & K Universal (Aldbrough, England) were used in the study. The animals were housed on a 12h light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by the Animal Ethical Committee of Gothenburg University. All efforts were made to minimize animal suffering and the number of animal used.

Retrograde tracing (Paper III)

Under sodium pentobarbital (50mg/kg, i.p.) anesthesia, 4% (dissolved in distilled water) Fluoro-Gold (FG) (Schmued & Fallon, 1986) was injected unilaterally (right side) into six different targets. For each investigated target, three animals were used. Briefly, injections of FG (1-1.5 μl each) were made into the submandibular salivary gland, the thyroid, as well as the cervical lymph nodes at 2-5 sites. Tracer injections, 3-4, were also done in the forehead skin. In the forepaw pad 1-2 injections of tracer were made into each paw pad tubercle, totally up to 10 sites. A total volume of 4 μl of tracer was injected into the anterior chamber of the eye after insertion of the needle into the lateral corner of the anterior chamber. Animals were allowed to survive for 4-5 days before sacrifice.

Tissue preparation (Papers II, III and IV)

Under sodium pentobarbital anesthesia, both normal and experimentally treated animals were perfused transcardially with 4% PF (pH 7.4). Different tissues, including various sympathetic ganglia (Papers II and III), the entire spinal cord (Paper IV), and the forepaw pad (Paper III), were then removed and post-fixed overnight in the same fixative and stored at 4°C in a PBS solution containing 0.1% sodium azide and 20% sucrose. The different specimens were frozen with compressed CO_2, sectioned in a cryostat at 10 μm, or at 6 μm in some cases, and mounted on gelatinized glass slides for immuno-histochemistry.

Immunofluorescence procedures

Primary antibodies

See table 1.

Secondary antibodies

Biotin-conjugated AffiniPure donkey anti-guinea pig IgG, dilution 1:200.

Biotin-conjugated AffiniPure donkey anti-rabbit IgG, dilution 1:200.

Biotin-conjugated AffiniPure donkey anti-goat IgG, dilution 1:200.

FITC-conjugated AffiniPure (FITC) donkey anti-rabbit IgG, dilution 1:50.

Table 1. The primary antibodies used in this thesis

Antibody	Species	Directed against	Source	Working dilution
anti-ChAT	rabbit	choline acetyltransferase	Chemicon Ab865	1:400
anti-ChAT	rabbit	human placental enzyme	Hersh L [1)]	1:400
anti-CgA	rabbit	chromogranin A	Fischer-Colbrie [2)]	1:400
anti-CgB	rabbit	chromogranin B, sequence 552-562	Fischer-Colbrie [3)]	1:800
anti-CgC (SgII)	rabbit	chromogrnain C (secretogranin II)	Fischer-Colbrie [4)]	1:1600
anti-CGRP	rabbit	synthetic calcitonine gene-related protein	Genosys, CA-O8-220	1:400
anti-galanin	rabbit	galanin	MILAB, A94	1:500
anti-GAP43	rabbit	dephospho- and phospho-	Wilkin GP [5)]	1:3000
anti-GFAP	rabbit	glial fibrillary acidic protein	Promega, G560A	1:800
anti-NeuN	mouse	purified neuronal nuclei from mouse brain	Chemicon, MAB377	1:200
anti-NESP55	guinea pig	NESP55, sequence 234-241 (GAIPIRRH)	Fischer-Colbrie [6)]	1:4000/1:1000
anti-NESP55	rabbit	NESP55, sequence 234-241 (GAIPIRRH)	Fischer-Colbrie [6)]	1:200
anti-NPY	goat	synthetic neuropeptide Y	ARPL [7)], NA1236	1:100
anti-NPY	rabbit	synthetic neuropeptide Y	Sigma, N9528	1:8000/1:4000
anti-secretoneurin (SN)	rabbit	chromogranin C, sequence 154-186	Fischer-Colbrie [4)]	1:800
anti-SP	rabbit	synthetic substance P	Amersham, RPN1572	1:800
anti-TGN38	mouse	trans-Golgi network, sequence 1-17	Oncogene, NB10	1:800
anti-TH	rabbit	purified tyrosine hydroxylase	Sigma-Aldrich, T8700	1:800
anti-TH	sheep	purified tyrosine hydroxylase	Chemicon, AB1542	1:800
anti-VIP	rabbit	vasoactive intestinal peptide	Theodorsson Elvar [8)]	1:100

1) (Li *et al.*, 1998a); 2) (Fischer-Colbrie & Schober, 1987); 3) (Kroesen et al., 1996); 4) (Kirchmair et al., 1993); 5) (Curtis *et al.*, 1993); 6) (Ischia et al., 1997). 7) ARPL: Affiniti Research Products Limited; 8) gift from E Theodorsson.

Texas Red-conjugated AffiniPure (TxR) donkey anti-rabbit IgG, dilution 1:50.

Texas Red-conjugated AffiniPure (TxR) donkey anti-mouse IgG, dilution 1:50.

Cy™5-conjugated AffiniPure (Cy5) donkey anti-mouse IgG, dilution 1:50.

Cy™5-conjugated AffiniPure (Cy5) donkey anti-sheep IgG, dilution 1:50.

Fluorescein (DTAF)-conjugated streptavidin, 1:800.

All the secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Immunofluorescence

For single immunofluorescence studies, samples (tissue sections or PF-fixed CAD cells) were incubated in normal donkey serum (1:50, Biotrend, Germany) for 1 hour to block any unspecific binding sties of the secondary antibodies, followed by incubation with different primary antibodies (table 1) over night. After rinsing the samples were incubated in Biotin-conjugated AffiniPure IgG, followed by incubation in Fluorescein conjugated streptavidin (DTAF). Finally, the samples were rinsed and mounted using an anti-fading mounting medium (Fluorescent mounting medium, DakoCytomation). The samples were analyzed under microscope and stored frozen.

For double immunofluorescence studies, samples were pre-incubated in normal donkey serum before incubation overnight in a mixture of two primary antibodies raised from different species (Table 1). After rinsing samples were incubated in a mixture of FITC and TxR conjugated secondary antibodies for two hours and mounted and analyzed. In some cases, Biotin-conjugated AffiniPure IgG was applied to replace FITC, followed by incubation in DTAT.

Triple immunofluorescence experiments, in which three primary antibodies from different species were used, was carried out to compare the distribution of three antigens. In this case Cy5 conjugated anti-IgG was applied as the secondary antibody, in addition to Biotin/FITC, and TxR conjugated anti-IgG.

CAD cells stimulated with potassium: The CAD cells in the first experiment (KCl, 60-120 mM, was added to the medium) were single-labeled with anti-NPY, followed by the secondary labeling with Biotin-conjugated AffiniPure IgG, then DFAT. The cells in the second experiment (KCl, 60-120 mM, as well as anti-NPY, were added to the medium) were immuno-stained directly with Biotin conjugated secondary antibody, followed by DTAF.

Confocal laser scanning microscopy

After immunofluorescence staining, the sections were examined in a confocal laser scanning microscope (CLSM) with a krypton/argon laser (Bio-rad, MRC 1024, Richmond, VA, USA) using single, dual or triple channel scanning. The excitation filter for FITC (488 nm), TxR (568 nm) and Cy5 (648) were selected. Colocalization of two or three markers was verified by merging the images from the two or three channels. Images were processed using Adobe Photoshop (Version 5.5).

Some images (Paper IV) were captured by a digital camera (Nikon D70) mounted on a fluorescence microscope (MICROPHOT-FXA, Nikon).

Western blot (Paper I)

CAD cells, grown in the SCM or the PFM, were counted with the use of a Burker Chamber and then homogenized. The pellets were lysed in a lysis buffer (1% NP-40, 10% Glycerol, 197 mM NaCl, and 20 mM Tris (pH 8.0)) containing Protease Inhibitor Cocktail Set III (Calbiochem) for 15 min on ice, then spun at 14000 rpm for 15min at 4 °C. The supernatant was collected, assayed for protein content according to Bradford (Bradford, 1976), and stored at –80°C. Aliquots of each sample containing equal amounts (20 μg) of proteins were diluted in NuPage LDS Sample Buffer (Cat. No. NP 0007, Invitrogen). NuPage Reducing Agent (Cat. No. NP 0004, Invitrogen) was added, followed by denaturation at 70 °C for 10 min. After electophoresis, proteins were transferred to polyvinyl difluoride (PVDF) membranes (Cat. No. 43660, BDH 4 Q Poole, UK) and then analysed by immunoblotting (Jakobsen et al., 2003). Rabbit polyclonal anti-NESP55 and anti-chromogranin A were diluted to 1:10000 and 1:2000, respectively.

Cell count and statistical analysis (Papers II and III)

The SCG, the stellate ganglion (SG) and mid-thoracic ganglia of rat were sectioned in their entirety and all sections were collected. Every tenth section (at least at 100 μm interval) was subjected to examination to avoid double counting of cells and totally 3-8 sections were selected for each ganglion.

The NESP55+, NESP55+/TH+ neurons, and the whole cell population in the ganglia were counted throughout the sections. The population size of NESP55 neurons in the different ganglia was calculated, as well as the presence of TH-IR in the NESP55+ neurons. The data were expressed as mean ± SD (Paper II).

Cells co-labeled with anti-NPY and anti-NESP55, as well as cells labeled with only one of the two antibodies, were counted throughout the SCG sections (4 male and 4 female). The ratio of co-expression of NPY-IR in the NESP55 positive cells, as well as of NESP55-IR in the NPY positive cells, was examined. The data were expressed as mean ± SD and compared between male and female rats using ANOVA (single factor). A p-value < 0.05 was considered statistically significant (Paper II).

FG was injected into the areas where the SCG and SG neurons were thought to project. This marker was, as demonstrated earlier (Li et al., 1998b) endocytosed by nerve terminals in the area and retrogradely transported to the parent neuron. All retrogradely labeled ganglion (SCG and SG) cells, clearly distinguishable from background levels and displaying a nucleus, were counted. The longest and shortest diameters of cells were measured with a calibrated eye-piece graticule. The average of these two values, referred to by Gibbins (Gibbins, 1991), was taken as a measure of cell diameter. Tracer labeled cells in the SCG and SG were classified, by their average diameter, into three types: small (< 25 μm), medium (25-30 μm), or large (≥ 30 μm). The presence of NESP55-, NPY- and CGRP-positive material in the labeled cells was recorded. Cells positive to both NESP55 and CGRP in the SG sections were counted, as was the clear presence of FG in these cells. All counts were presented as uncorrected numbers. (Paper III).

RESULTS

NESP55-IR in the CNS-derived CAD cell line (Paper I)

NESP55-IR, like other chromogranins/peptides such as CgA, CgB, SgII, NPY, SP, VIP, and galanin, was present in the cell body and the processes of CAD cells, with a typical granular pattern. However, NESP55-IR was also observed in the fine short processes, extending from the cell body, as well as from the long processes, in a fringe-like manner. Such distribution was sometimes also noted for VIP and SP (Fig. 2 in paper I). GAP43-IR, a protein highly associated with outgrowth of neurites and development (Jacobson et al., 1986), was also always observed in these fringe-like structures, partially overlapping with NESP55-IR (Fig. 3 in I).

By western blot, both NESP55-IR and CgA-IR were observed to be up-regulated in CAD cells after differentiation (Fig. 7 in I).

The potassium stimulation experiments were carried out with the differentiated CAD cells to investigate whether CAD cells were capable of endocytosis/exocytosis. However, no sign of endo/exocytosis could be observed (see discussion part).

NESP55 was expressed in various sympathetic ganglia (Papers II)

The expression of NESP55 in postganglionic sympathetic neurons of rat was investigated. NESP55 positive neurons represented approximately 13-19% and 6-9% of the total population of neurons in the SCG and the SG, respectively (Table 1 in II). These NESP55 positive neurons were scattered throughout the entire ganglion. All NESP55+ neurons in the SCG were TH positive, thus, noradrenergic. However, only 75-90% of the NESP55+ neurons in the SG were noradrenergic. In the thoracic sympathetic chain ganglia, NESP55 positive neurons were present in small groups, representing 3-7% of the whole cell population. The majority (70-85%) of these NESP55+ neurons were apparently non-noradrenergic (TH negative). By double labeling with anti-NESP55 and -NPY, we observed in the SCG that about 23% (male), or 25% (female) of all NPY+ neurons appeared to contain NESP55-IR, while 80% (male), or 87% (female) of the

NESP55+ neurons were also NPY positive. The relative frequency of NPY-IR present in the NESP55 positive SCG neurons in the female, 87%, was slightly higher than that observed in the male (80%). This difference was statistically significant (Fig. 6 in II).

No nerve terminals/varicosities labeled by anti-NESP55 were seen in these ganglia.

The NESP55 immunofluorescent material was observed to be clearly concentrated in the perinuclear region with a granular appearance in postganglionic sympathetic neurons (Fig. 5 in II).

NESP55 positive sympathetic neurons projected to a number of peripheral organs (Paper III)

A number of rat tissues were subjected to a retrograde tracing study. The submandibular gland, the iris, the forehead skin, the cervical lymph nodes and the thyroid were chosen as potential targets for the SCG neurons, while the forepaw pad was considered target of the SG neurons. SCG neurons were found to ipsilaterally project to the submandibular gland, the lymph nodes, the thyroid, as well as the iris, and bilaterally to the forehead skin. The cell size of retrogradely labeled neurons varied from small to large. In general, the majority of neurons projecting to the submandibular gland displayed large cell bodies. In contrast, the neurons projecting to the thyroid and the forehead skin were always of small size. Among neurons projecting to the submandibular gland, the cervical lymph nodes and the iris, less than 10% contained NESP55-IR; of the forehead skin-projecting neurons, 36% were NESP55 positive; while no thyroid-projecting neurons contained NESP55-IR (Table 1 in III). All NESP55 positive forehead skin- and iris-projecting neurons also contained NPY-IR, a peptide with autonomic effects, for instance, vasoconstriction (Gibbins, 1992; Gibbins, 1995). However, a subset of NESP55+ submandibular gland- and cervical lymph node-projecting neurons was clearly devoid of NPY-IR (Fig. 2 in III).

The SG neurons were found to project ipsilaterally into the forepaw pad. Of the 89 labeled neurons projecting to the forepaw pad, 79 (89%) contained NESP55-IR. Double

labeling experiments revealed that NPY-IR was exclusively present in NESP55 containing tracer labeled neurons, which always had small or medium cell bodies. Likewise, CGRP-IR, with sudomotor effects in the autonomic system (Landis & Fredieu, 1986; Anderson et al., 2006), was present only in NESP55 positive labeled neurons, which were always of large size. NPY+ and CGRP+ neurons represented 19% and 30% of the total number of the labeled cells, respectively. CGRP+ neurons appeared devoid of NPY-IR and *vice versa* (Fig. 3 in III). Thus, four subpopulations of the forepaw pad-projecting SG neurons were classified by their components of peptides: 1. NESP55+/CGRP+/NPY-, 2. NESP55+/CGRP-/NPY+, 3. NESP55+/CGRP-/NPY-, 4. NESP55-/CGRP-/NPY-.

Among the NESP55+/CGRP+ neurons in the rat SG, only 44% were found to be retrogradely labeled from the forepaw pad.

No NESP55-IR was detected in the nerve terminals around the sweat glands despite the observation that approximately 90% of the forepaw pad projecting neurons contained NESP55-IR. However, strongly fluorescent nerve terminals with CGRP-IR were present around the secretory acini (Fig. 4 in III).

NESP55-IR was present in various types of neurons in the spinal cord (Paper IV)

NEPS55-IR in autonomic neurons

NESP55-IR was detected in preganglionic sympathetic neurons in the present study. The intermediolateral (IML) cell column contained multipolar nerve cells with NESP55-IR at the thoracic and lumbar spinal cords. In addition, NESP55-IR was also observed in the central autonomic nucleus and other sympathetic neurons distributed between the central autonomic nucleus and the IML. Parasympathetic neurons in the sacral spinal cord appeared to contain comparatively weak NESP55 immunofluorescence (Fig. 3 in IV).

The NESP55 immunofluorescent material was clearly concentrated in the perinuclear region with a granular appearance in these sympathetic neurons (Fig. 8 in IV).

NESP55-IR in motoneurons

NESP55-IR was present in different motoneuron columns (Molander et al., 1984; Molander et al., 1989) throughout the whole spinal cord where the immunofluorescent material, present with a dust-like appearance, was evenly distributed in the whole cytoplasm and extending into dendrites as well as axons (Fig. 2 in IV). The most prominent staining was found in neurons at the lower lumbar level and the lumbar-sacral transition area where sexual dimorphism has been observed (Schroder, 1980). NESP55-IR appeared to be widespread in most motoneurons. However, when colocalizion studies were carried out using anti-ChAT as a marker of cholinergic motoneurons, we found that not all ChAT+ large neurons contained NESP5-IR, but a small population of ChAT+ cells, with the appearance of motoneurons, appeared NESP55 negative. On the other hand, some NESP55+ neurons, resembling motoneurons morphologically, appeared devoid of ChAT-IR (Fig. 6 in IV).

NESP55-IR was also detected in the intermingled dendritic processes, as well as in the axons of motoneurons, entering the ventral root (Fig. 2 in IV). However, NESP55+ nerve terminals were never observed.

NESP55-IR in other types of spinal neurons

NESP55-IR was present also in some spinal neurons that were of small size in comparison with motoneurons, as well as with sympathetic neurons. These neurons were scattered throughout most spinal laminae with the exception of laminae I-III. They probably represent interneurons (Li et al., 1999). Double labeling with anti-NESP55 and anti-ChAT revealed a colocalization in some of the interneurons, but interneurons expressing only one of the two proteins could be seen also.

Moreover, NESP55-IR was detected in a group of neurons located ventral to the dorsal horn in the dorsolateral funiculus (Fig. 4 in IV), possibly corresponding to the lateral spinal nucleus (LSN) as described by Gwyn and Waldron (Gwyn & Waldron, 1968). The neuronal nature of these cells was suggested by the nuclear staining with a neuronal nuclear marker, NeuN (Mullen et al., 1992).

The sensory dorsal root ganglion of the rat was devoid of NESP55-IR.

Comparison between the intracellular distribution of NESP55-IR in motoneurons and sympathetic neurons (Papers II, III, IV)

As discussed in the above sections, the intracellular distribution of NESP55-IR differed between neuronal types. The immunoreactive material was concentrated in the perinuclear region in both preganglionic and postganglionic sympathetic neurons, showing a typical granular structure (Fig. 5 in II; Fig. 8 in IV;). In contrast, in spinal motoneurons NESP55-IR was evenly distributed throughout the whole cytoplasm in small dust-like particles (Fig. 7, 8 in IV).

To further illustrate this observation, we carried out the double labeling experiments. CGRP is an established peptide located in the LDCVs of both CNS and PNS neurons, showing a perinuclear (trans-Golgi netwok) staining immunohistochemically. Double labeling with anti-NESP55 and -CGRP revealed that NESP55-IR largely overlapped with CGRP-IR in the perinuclear region in SG neurons (Fig. 8 in II). However, in spinal motoneurons, the diffuse distribution of NESP55 was clearly distinct from the perinuclear staining of CGRP (Fig. 7 in IV). In agreement, double immunostaining with anti-NESP55 and -TGN38, the trans-Golgi network marker, showed them to overlap in the autonomic sympathetic neurons (Fig. 5 in II; Fig. 8 in IV), but they were clearly separated in the motoneurons (Fig. 8 in IV).

DISCUSSION

Methodological consideration

Specificity of the NESP55 antibody

Immunofluorescence is widely applied to study distribution and localization of proteins in various tissues. The quality or specificity of an antibody raised against an epitope of a particular protein is the core element in the experimental protocol. Anti-NESP55 used in the present study is a kind gift from Dr. Reiner Fischer-Colbrie at the department of Pharmacology, University of Innsbruck, where NESP55 was first characterized and the antiserum was produced (Ischia et al., 1997). The antiserum was prepared against a synthetic octapeptide (GAIPIRRH) representing the C-terminus (amino acids 234-241) of NESP55, and generated by coupling the synthetic peptide via an extra N-terminal cysteine to maleiimide activated keyhole limpet hemocyanain (or hemocyanin). For experiments the final bleed was affinity purified. This antibody recognizes both the free peptide GAIPIRRH/GPIPIRRH and the intermediate- and large-sized peptides including the precursor NESP55 equally well (Fischer-Colbrie *et al*., 2002).

The antiserum stains a single band of Mr 55000 on Western blots (Ischia et al., 1997). In brains of NESP55 knockout mice no positive staining was seen with the antiserum (Plagge et al., 2005). Immunohistochemical staining of NESP55 in rat sympathetic ganglia (Paper II) and the spinal cord (Paper IV) was completely abolished by preadsorption of the antiserum with the peptide used for immunization. Furthermore, there was a complete match of the neuronal immunohistochemical staining pattern in the rat brain and spinal cord with the mRNA distribution established by *in situ* hybridisation histochemistry (Bauer et al., 1999a).

Potassium stimulation of CAD cells and immunofluorescence

The CNS-derived CAD cell line was derived from a mouse CNS tumor, possibly originating from the locus coeruleus. CAD cells cultured in the PFM undergo a differentiation towards a neuronal phenotype, with very long neurite-like processes. The differentiated cells express many neuron-specific proteins, as well as glia proteins (Li et

al., 2007). A number of receptors and neuropeptides, including NESP55 (Paper I), was upregulated in CAD cells after differentiation (Hashemi et al., 2003).

The original project was to characterize the CAD cell line as a potential model to study axonal transport. Although we turned to other directions later it is necessary to describe the design of the potassium stimulation experiments. Potassium stimulation was performed by adding KCl (60-120 mM) to the cell culture medium for different time periods to investigate if CAD cells were capable of endocytosis/exocytosis. If CAD cells were able to release the matrix peptides in response to acute high potassium stimuli, the visible fluorescence intensity of NPY, which is abundantly present in the CAD cells, should decrease with time of stimulation. The second experiment was performed based on the following idea: If exocytosis occurs, the granule matrix would be exposed to the anti-NPY containing medium. The anti-NPY would attach to the exposed remaining core of the releasing granules and be retrieved in the granule during endocytosis. Thus, the presence of anti-NPY, demonstrated by incubation with the secondary antibody only, would demonstrate that endocytosis had occurred. However, in our present study no evidence for endocytosis/exocytosis could be observed.

Release of NPY in primary SCG cultures has already been demonstrated using this method in our lab (Li & Dahlstrom, 2007). However, we failed to show the same results in CAD cells. Possible explanations are that the method may not be an optimal approach to study CAD cells, or the pathway by which CAD cells release their matrix peptides is different from the neuronal cells, or CAD cells are not capable of endocytosis/exocytosis at all.

Retrograde tracing

To investigate the peripheral projections of NESP55 positive sympathetic neurons we performed retrograde tracing experiments. To minimize dye leakage, the injecting device, Microliter Syringe (Hamilton Bonaduz, Schweiz), was left in place for 1-2 minutes after injection of FG into various organs. Any leakage after this time was soaked up with a cotton pad to make sure that tracer was restricted to the tissues of interest.

Because only three rats were recruited in every experimental group, the number of labeled cells from different targets, especially in the iris group, where only 39 neurons were retrogradely labeled, was relatively low. It should be kept in mind that the number of retrogradely labeled cells observed in the present study probably represents only a fraction of all neurons projecting to a certain tissue. Presumably, the larger the target, the more incomplete the diffusion of the injected tracer, and the less likelihood that all projecting neurons will be labeled. Thus, the raw figures we report in the present study provide qualitative, not quantitative, information.

NESP55 may be involved in cell adherence?

NESP55-IR, like other chromogranins/peptides studied, was present in the cell body and the processes of the differentiated CAD cells. Interestingly, we observed that NESP55-IR also appeared in thin fringe-like processes, partially overlapping with GAP43-IR. This observation, apparently not noted for other chromogranins, is striking. The role of NESP55 in this structure is unknown. However, NESP55 may be involved in cell adherence or share a function related to that of GAP43. To further understand the CAD cells, as well as NESP55, experiments at an ultrastructural level are necessary to investigate the cellular organelles in these fine processes. Also, it would be of interest to compare the effect of different coating of the culture plates.

NESP55 may have a functional role in some populations of sympathetic neurons.

The sympathetic nervous system and the parasympathetic nervous system, to a certain extent antagonists, plus the enteric nervous system, form the autonomic nervous system (ANS), controlling autonomic activities of all organs and tissues of the body, except the skeletal muscle fibres. In the sympathetic nervous system, signals from the preganglionic neurons located in the brain stem and the spinal cord, in turn coordinated from higher centers in the CNS, are relayed, distributed, and integrated in paravertebral, prevertabral and intramural ganglia. Here the signals effecting the postganglionic neurons are further modified and delivered to the neuroeffector junctions to regulate various end-organ responses. The parasympathetic nervous system also has preganglionic and post-

ganglionic neurons, located in the cranial and sacral region of the spinal cord, and in ganglia close to, or in the wall of, the innervated tissues (Gabella, 1976; McLachlan, 1995). In our present study NESP55-IR was present in the central autonomic nucleus, the IML, as well as in the lateral horn of the sacral spinal cord, suggesting that NESP55 may be involved in the preganglionic regulation of both the sympathetic and parasympathetic nervous systems.

NESP55-IR was also detected in a subpopulation of the principal neurons in the SCG, SG, the thoracic chain ganglia, and the celiac ganglion of the rat, which inspired us to explore whether NESP55 is involved in a specific function in these tissues. Sympathetic neurons commonly utilize a "classic" neurotransmitter and one or more peptides to provide precise regulation of specific peripheral effectors, In other words, the autonomic pathways are "chemically coded" (Gibbins, 1995). By retrograde tracing, we found that a subgroup of NESP55-IR neurons in the SCG projected to the submandibular salivary gland, the cervical lymph nodes, the iris and the forehead hairy skin. However, the thyroid-projecting neurons lacked NESP55-IR.

NPY is thought to be a neuromodulator inducing vasoconstriction, prejunctional inhibition of norepinephrine release and postjunctional potentiation of norepinephrine effects (Gibbins, 1992; Benarroch, 1994). Coexistence of NESP55 and NPY in the neurons projecting to the above-mentioned organs, as observed after the double-labeling experiment, was evident, suggesting that NESP55 may be involved in their vascular regulation. NESP55 and the precursor of NPY (pro-NPY) share the same enzyme, PC2, to produce smaller peptides, for instance, GAIPIRRH and NPY (Paquet et al., 1996; Fischer-Colbrie et al., 2002). Thus, metabolism of NESP55 may lead to an inhibited production of NPY by competing with the co-stored pro-NPY for the PC2, as proposed by Seidah and colleagues (Seidah et al., 1987). In addition, a subgroup of NESP55-IR neurons projecting to the salivary gland and the lymph nodes apparently lacked NPY-IR, suggesting that NESP55 is also involved in other autonomic activities, such as secretomotor effects. Neurons in the rat SCG, which lack NPY-IR, have been suggested to be secretomotor neurons (Gibbins, 1995).

Sudomotor neurons express neuropeptides CGRP and VIP, in addition to the classic neurotransmitter acetylcholine (Gibbins, 1995). Colocalization of NESP55- and CGRP-IR was found in the forepaw pad projecting neurons in the present study, suggesting that NESP55 may also be involved in the sudomotor regulation of the sweat glands in the forepaw pad. Approximately 56% of NESP55+/CGRP+ neurons in the SG were, however, not labeled by the retrograde tracer FG. It is possible that these neurons were sweat gland projecting neurons, which were not labeled by FG. Alternately, it is also possible that these neurons have functions other than sudomotor effects.

NESP55 cannot be observed in nerve terminals by immunohistochemistry.

No nerve terminals have been immunohistochemically labeled by anti-NESP55 as observed in the present study and other studies (Li et al., 2002). The spinal motoneurons of the rat apparently contained NESP55-IR (Paper IV) but their endings, the motor endplates, appeared devoid of NESP55-IR (Li et al., 2002); the IML neurons expressed abundant NESP55-IR (Paper IV) but no NESP55+ nerve terminals were seen in any of the sympathetic ganglia, the projection targets of IML neurons (Papers II, III); NESP55-IR was clearly present in various sympathetic ganglia (Papers II, III) but absent from their autonomic nerve terminals, for instance, in the heart (Li et al., 2002), and the salivary gland (personal observation). Moreover, we could not detect any NESP55-IR in the forepaw pad, despite the observation that approximately 90% of forepaw pad-projecting neurons contained NESP55-IR (Paper III).

This morphological absence of NESP55 immunoreactive material in nerve terminals, was also noted for another chromogranin family member, CgA. CgA was found to be widely distributed in most neurons in the pelvic ganglia projecting to the vas deferens, but absent from the nerve terminals of this organ (Li et al., 1998b). Similarly, CgA was present in the somatic motor perikarya in the rat spinal cord, but very sparse in the motor endplates (Li & Dahlstrom, 1992; Li et al., 1992). Thus, both CgA and NESP55 are peptides present at a high level in cell bodies, but barely detectable, or apparently absent, in nerve terminals. A possible explanation for this is that the concentration of antigen may be

below the detection level of immunohistochemistry in the peripheral terminals, which is supported by the observation that very small levels were measured by RIA in these tissues (Li et al., 2002). Alternatively, posttranslational modification (e.g. proteolytic processing) of the peptides during axonal transport may render the peptide undetectable by the antiserum used. Also, a secretion of NESP55 or its fragments into surrounding, non-neuronal, tissue may have taken place.

Secretion of NESP55 may be cell type-specific.

Newly synthesized proteins leave the endoplasmic reticulum (ER) and pass through the TGN where they are sorted and packaged into different vesicles destined for cellular export and secreted in either a constitutive or a regulated manner by exocytosis. The constitutive secretion is a simple function occurring in every cell type where proteins are continuously secreted to the exterior. In contrast, the regulated secretion takes place only in response to stimulation in specialized cells. Proteins stored in LDCVs or in secretory granules are secreted from cells or cell processes via the regulated pathway in response to stimulation. A third secretory pathway, constitutive-like secretory pathway, was also proposed for proteins originally sorted into secretory granules. However, they escape from the immature secretory granules before maturation, and may be constitutively secreted into the extracellular environment (Arvan & Castle, 1998; Taupenot et al., 2003).

The constitutive secretion of NESP55 has been demonstrated in the AtT-20 cells (Eder et al., 2004). Fischer-Colbrie (Fischer-Colbrie et al., 2002) also proposed this pathway for NESP55 in the sciatic nerve, as well as in the brain. In the present study we found that both NESP55 and CGRP were present in motoneurons, but with a strikingly different pattern, where CGRP alone colocalized with TGN38 while NESP55 was more diffusely spread in the whole cytoplasm. This clearly indicates that NESP55 and CGRP may be stored in different types of vesicles, i.e. CGRP in the putative LDCVs, but NESP55 probably in smaller particles. Thus, constitutive secretion of NESP55 may occur also in the spinal motoneurons. Constitutively secreted proteoglycans in the brain, such as agrin secreted from motor axons (Gautam et al., 1996) and reelin secreted from Cajal-Retzius

cells (D'Arcangelo et al., 1995), have been characterized, and shown to be part of the extracellular matrix with implications for neuronal guidance and development, and maintenance of neuronal circuitry.

In contrast, in the sympathetic neurons NESP55-IR appeared to be concentrated in the perinuclear region, overlapping with CGRP-IR and TGN 38-IR, implying that NESP55 is located in the LDCV in these neurons. In agreement, NESP55 was previously demonstrated by tissue fractionation followed by RIA to be distributed in the LDCV/hormone storage vesicles in the adrenal medulla and splenic nerve of the cow (Ischia et al., 1997; Leitner et al., 1999). All the evidence seem to support the idea that NESP55, in these tissues, may be released via the regulated pathway.

Does NESP55 posses the functions of the "classic chromogranins"?

The most discussed function of Chromogranins is their involvement in the formation of secretory vesicles (Ozawa & Takata, 1995). Whether or not NESP55 shares the same functional feature with its siblings is yet unknown. However, based on the information obtained from previous studies, such a function cannot be excluded. Our present study revealed that NESP55-IR is tightly associated with the TGN, the main compartment for the formation of secretory granules/LDCVs, in sympathetic neurons, providing additional evidence that NESP55 may have an intracellular role like other chromogranins in these neurons.

Another putative function of chromogranins is acting as a precursor of small bioactive peptides. NESP55 was thought to be the precursor of the tetrapeptide LSAL, an endogenous antagonist of the serotonergic 5-HT_{1B} receptor, when it was first characterized from bovine adrenal medulla. However, later studies showed that LSAL was mutated to LHAL as observed after cloning of the man, rat and mouse homologues (Hayward et al., 1998b; Weiss et al., 2000). Whether LHAL acts as a modulator of serotoninergic system like LSAL is not unknown. But it is also possible that there is a different gene coding for the precursor of LSAL, arguing against the role of NESP55 as the sole precursor for LSAL. However, NESP55 is indeed a precursor for smaller fragments as demonstrated by

its processing into small peptides like GAIPIRRH/GPIPIRRH in tissues to a varying degree (Ischia et al., 1997; Lovisetti-Scamihorn et al., 1999a; Lovisetti-Scamihorn et al., 1999b; Li et al., 2002), although the functional significance of GAIPIRRH/GPIPIRRH has not been characterized.

Other functional implications of NESP55

Chromogranins, especially CgA, CgB and SgII, are present in much higher amounts in endocrine, neuroendocrine, and neuronal tumors than in normal tissues (Winkler & Fischer-Colbrie, 1992). Thus, chromogranins are considered to be important indicators for diagnosis of the neoplasms. Measurement of chromogranin levels in blood can be used to distinguish between malignant and benign diseases, as well as to monitor the progression or regression of neuroendocrine tumors during treatment (Taupenot et al., 2003). In comparison, the distribution of NESP55 in endocrine and neuroendocrine tissues is more limited in both normal and pathological conditions. NESP55 is expressed in endocrine tumors of pancreas, adrenal medulla, and, sparsely, in gastrinomas and nonfunctioning endocrine pancreatic tumors. However, NESP55 is not expressed in ileal carcinoids or adrenocortical adenomas. Thus, NESP55 can be used to identify subtypes of neuroendocrine tumors (Jakobsen et al., 2003; Nilsson et al., 2004).

A role in controlling exploratory behavior was previously suggested for NESP55. A series of behavioral tests were conducted in a NESP55-knock out mouse model (Plagge et al., 2005). These NESP55 deficient mice developed without obvious phenotype effects and were fertile. However, the mice showed increased excitement towards a novel environment but spent less time to explore the new surroundings.

Imprinted genes are of importance in the regulation of placental development, fetal growth, and neurodevelopment (Davies et al., 2005; Fowden et al., 2006). Loss of imprinting has been recently observed in several cancer cells (Cui et al., 1998). There are few data available for NESP55 in these areas so far. However, recent studies demonstrated that genomic organization of NESP55 gene was critical in the development

of pseudohypoparathyroidism type 1b (Freson et al., 2002; Bastepe et al., 2005), a disorder of renal parathyroid hormone (PTH) resistance.

Significance of this work and future directions

NESP55 is the youngest member of the chromogranin family. Comparatively limited data are so far available for this novel protein. Our present study provides information about NESP55 in terms of its neuronal distribution and intracellular localization, as well as the peripheral projections of NESP55 containing postganglionic neurons. It is hoped that this may provide the basis for a better understanding of the functional significance of this protein.

There are several interesting questions raised during the present study, which remain to be resolved in the future. The difference between the intracellular distribution of NESP55-IR in the motoneurons and the sympathetic neurons is striking. However, to clarify the exact subcellular localization of NESP55 in these neurons, as well as in the CAD cells, especially in the fine fringe-like processes, further studies, such as sucrose fractionation followed by RIA or electron microscopy, are necessary. Moreover, concerning the abundant expression of NESP55 in the SG neurons, it would be of interest to know if NESP55, as an imprinted gene, is involved in the development of SG neurons projecting to the sweat glands. Sweat gland projecting neurons in the SG are believed to switch neurotransmitter phenotype from noradrenergic to cholinergic in the late embryonic stage, or postnatally (Leblanc & Landis, 1986; Landis et al., 1988; Asmus et al., 2000). Thus, it would be interesting to investigate the appearance/expression of NESP55 in the SG during different developmental stages.

CONCLUSIONS

I. The CAD cell line expresses, under our culture condition, both glia and neuron specific proteins, including a number of neuropeptides, suggesting that the CAD cell line may be suitable for various neurobiological studies. The expression of NESP55 in the fine fringe-like processes of the differentiated CAD cells is characteristic and unique, implicating that NESP55 possibly may have a role in cell adherence.

II. NESP55 was expressed in different sympathetic ganglia including the SCG, the SG, the sympathetic chain ganglia, and the celiac ganglia of rat. NESP55 containing cells in the SCG projected to the submandibular gland, the iris, the forehead skin, and cervical lymph nodes, but not to the thyroid. In the SG, the NESP55 containing neurons projected to the forepaw pad. Our findings suggest that NESP55 may have a functional role in some populations of sympathetic neurons, probably neurons with vasoactive, sudomotor or secretomotor effects.

III. NESP55-IR was present in various types of neurons in the rat spinal cord, including the motoneurons, autonomic neurons, interneurons, and the LSN, suggesting that NESP55 may be involved in functions of multiple types of neurons at the spinal cord level.

IV. NESP55-IR small particles were diffusely present in large amounts throughout the cytoplasm of motoneurons, differing from its distribution in autonomic neurons, in which NESP55-IR, located in large granules, was mainly concentrated in the Golgi region. The data suggest that NESP55, in motor and autonomic neurons, may be sorted into different types of vesicles and possibly secreted via different routes, the constitutive or regulated pathway, respectively. Moreover, NESP55 may play a role in the formation of the LDCVs in sympathetic neurons, while in motoneurons, a role in the formation of the intra/extracellular matrix is speculated.

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APPENDIX: Papers I-IV